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The attached documents are exact copies of the European patent application described on the following page, as originally filed.

Les documents fixés à cette attestation sont conformes à la version initialement déposée de la demande de brevet européen spécifiée à la page suivante.

Patentanmeldung Nr. Patent application No. Demande de brevet n°

00306004.3

Der Präsident des Europäischen Patentamts;
Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets
p.o.

I.L.C. HATTEN-HECKMAN

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Anmeldung Nr.: Application no.: Demande n°: 00306004.3

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Anmelder: Applicant(s): Demandeur(s):
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Bezeichnung der Erfindung: Title of the invention: Titre de l'invention:
Method for detecting a variation of GH1 as indicator of growth hormone dysfunction

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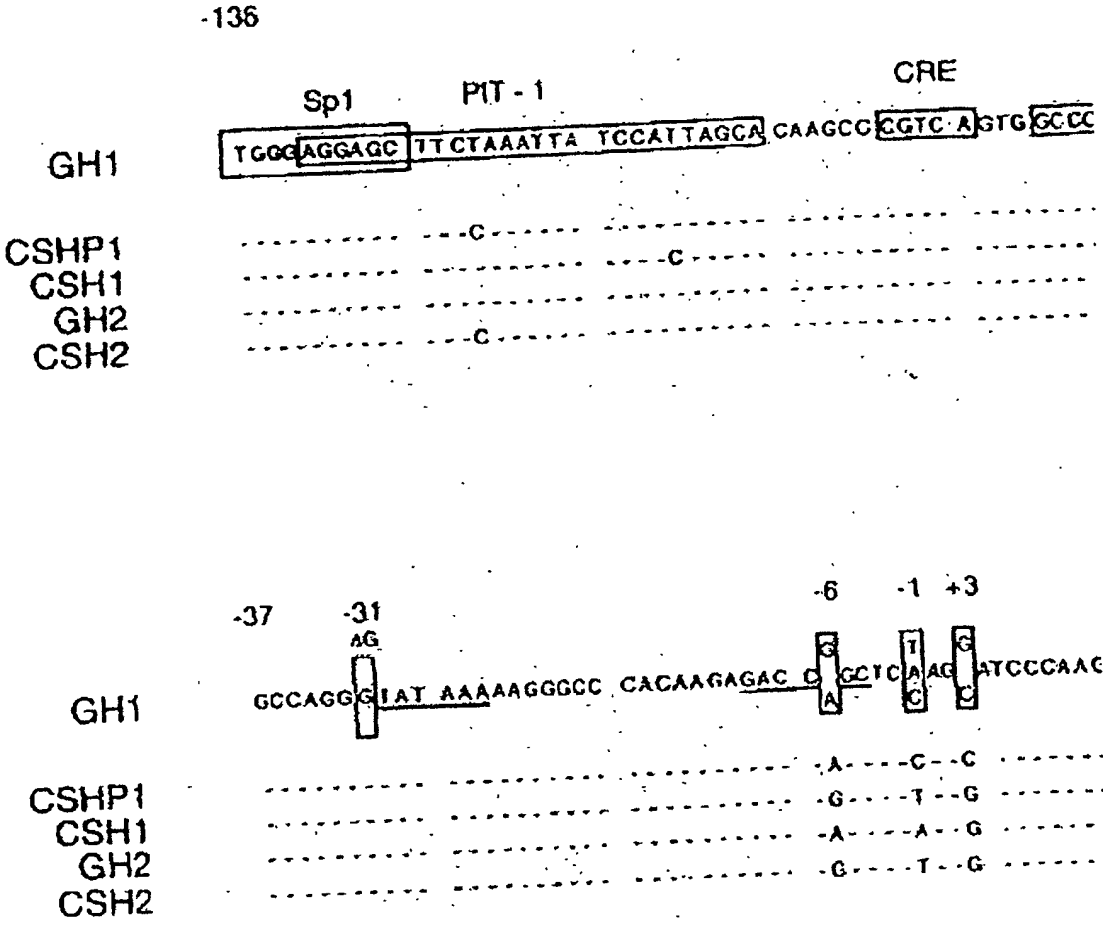

Fig. 3 Structure of the 5' untranslated region and promoter region of the human *GH1* gene. *Horizontal boxes* denote known, putative or inferred binding sites for transcription factors. *Vertical boxes* indicate polymorphic sites in the human population (data from Giordano et al. 1997; Wagner et al. 1997). The numbering scheme is by reference to the transcriptional initiation site at +1. The TATA box, a Chi-like element and the ATG translational initiation site are *underlined*. The human *GH1* promoter is also aligned with the promoters of the human *CSHP1*, *CSH1*, *GH2* and *CSH2* genes in order to indicate both the extent of homology but also the differences evident between promoters

```
1     GATCTTGTCTCAGAAAAACCCCAGAAAACAACAACAAAAAAACGCGCTAT
51    TGGGCTTATTTCTATTTTACGAAAATGAGGAACCTTGGTCACTGAGAGGT
101   TAAGAATGGGTGTTGCCTCATGGGTGCTGTTGGCATGTGGGGCAGGGAAC
151   TCTGCTTTGCCAGCCAATGCCCCTCCCGCCCCAGGCACCCACCGGTCATT
201   GAGACCAGCGCGATTGCCCTGATGCATGTGCAGACGCTCCCTCCGTTGGG
251   GCAGAGCCACCCTTAGTAGAGAACCACTGGTAACCTGGGGAGGTTTCCTG
301   GCTGCTACACTGCTGGGTCAGGACTCAAACCCACGTCCTTCTTAGCAGGT
351   CTTTTCCCAGTCACAGCAGCCCAGCAGATCCAGGACACCTGGGCTGGAGA
401   AGGAGGCAGGGGTCTGAGCTCTTCTTTCCCTGAGGTTCTTCCCCCTCCCG
451   GCATCCTAGGGTGGCTGGGGGTCGCTGCCTTCTTGCTTCCCCACCCCCTG
501   AGCCCCTTGTCCCTTCGCCCGACCCTGGCCCCCATTCCTTATTTTGATGT
551   TGCCTTCCTGGAAAGGGAGGTGTCGTCAGAAGTCAGAATGTGAAGCCCCG
601   GGCAGCCGAGGGCAGAGGCAGGGGTAGGCTTGTGAGGCCTCCTGTTCTGG
651   GAAGAAAAGGGCATCATGAGCCCAAAATGGGGCTCAGGGCTGTTGTCACC
701   AGTGAGGGAACAGACGGCTCAAAATGTGACAAATAACCCTGCAGGCGGTG
751   GGGCCCCCGCTGCCTCCGCCCCTCCTGAAAACAGATCCCTGCCCGGCATG
801   AATGGGAATGAAGCCCAAAGGCCACATCTGCATCCTTGTCCACGGGATGT
851   GTGTGTCTTCCCTCCCTGGAGTGGGAGCTGCTTCTCACTTGTTTTTTTAA
901   ATTTTTTATTTCAATATTTTCTGGGGTACAGGTAGTTTTTTTGGTTACCT
951   GGATAAGTTCTTTAGTGGTGATTTCTGAGATTTTAGCGCACCTGTCACCT
1001  GAGGATGGGTGCCAACTGCCCCCCACCAACCTCCGTGCCCCCTACAGTCC
1051  GCTGAGCCCTAGGACCAGCTCCAGAGTAGGCCCCACACCACAGGGTCCAG
1101  GCAACACACCTGTGTGAGCATGCACATACACACACACACACCCCCCAC
1151  AAACACGTGTGCCCCAAGCCTTTCCCAGTTATACCCCCCAACTTTGGGGA
1201  GACACTAGCCCCAAAGTTAATGAAGGACTCTGTCGTTAGGGGCTCAGGAA
1251  GAGTATTTCCTAAACCATGAGACTCCCAGATTTTGCCCCACTCCCCCCGG
1301  GTCAGTCTCTCTCCAGCCACCCTCACCAGCATGCGGGCCCATGGGCCTCA
1351  AGCTGACCTCAGGTGATGTTTATATTTCTGAGCTGTTTATTCCATGAACT
1401  GAACATCTGACAGCTTTTCAGAGAAATGTTTTTTCATTTGGAACATCTGG
1451  AAACAAGAAAGAACATCTGGGGCTGCCCGGAACGGGCTGTTCCTCGGATG
1501  AAACCTTAACCCTCCTGCCCCTGACTTCCGTGGCTCAGGTCCTGGCCTGC
1551  ACCCCTTGAGAGTGGCCCCCACCTTATCAGGTCCGAGGCCTAGGCCAAGA
1601  TC
```

Figure 4

```
      -700  ctgtttcttg gtttgtgtct ctgctgcaag tccaaggagc tggggcaata  -651
 5    -650  ccttgagtct gggttcttcg tccccaggga cctgggggag ccccagcaat  -601
      -600  gctcagggaa aggggagagc aaagtgtggg gttggttctc tctagtggtc  -551
      -550  agtgttggaa ctgcatccag ctgactcagg ctgacccagg agtcctcagc  -501
      -500  agaagtggaa ttcaggactg aatcgtgctc acaaccccca caatctattg  -451
      -450  gctgtgcttg gccccttttc ccaacacaca cattctgtct ggtgggtgga  -401
10    -400  ggttaaacat gcggggagga ggaaagggat aggatagaga atgggatgtg  -351
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      -300  caggttggcc accatggcct gcggccagag ggcacccacg tgacccttaa  -251
      -250  agagaggaca agttgggtgg tatctctggc tgacactctg tgcacaaccc  -201
      -200  tcacaacact ggtgacggtg ggaagggaaa gatgacaagc caggggcat   -151
15    -150  gatcccagca tgtgtgggag gagcttctaa attatccatt agcacaagcc  -101
      -100  cgtcagtggc cccatgcata aatgtacaca gaaacaggtg ggggcaacag   -51
       -50  tgggagagaa ggggccaggg tataaaaagg gcccacaaga gaccagctca    -1
        +1  aggatcccaa ggcccaactc cccgaaccac tcagggtcct gtggacagct   +50
       +51  cacctagcgg caATGGCTAC AGgtaagcgc ccctaaaatc cctttgggca  +100
20    +101  caatgtgtcc tgaggggaga ggcagcgacc tgtagatggg acgggggcac  +150
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      +251  aaaaacaaac agctcctgga gcagggagag tgctggcctc ttgctctccg  +300
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      +551  cgctgggaaa taagaggagg agactaagga gctcagggtt tttcccgaag  +600
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      +651  acaatgggag ctggtctcca gcgtagacct tggtgggcgg tccttctcct  +700
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      +951  CAGTCGTGGC TGGAGCCCGT GCAGTTCCTC AGGAGTGTCT TCGCCAACAG +1000
     +1001  CCTGGTGTAC GGCGCCTCTG ACAGCAACGT CTATGACCTC CTAAAGGACC +1050
     +1051  TAGAGGAAGG CATCCAAACG CTGATGGGGg tgagggtggc gccaggggtc +1100
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617

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Figure 5 (continued

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Anmelder: Applicant(s): Demandeur(s):

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Bezeichnung der Erfindung: Title of the invention: Titre de l'invention:

Method for detecting a variation of GH1 as indicator of growth hormone dysfunction

In Anspruch genommene Priorität(en) / Priority(ies) claimed / Priorité(s) revendiquée(s)

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Bemerkungen: Remarks: Remarques:

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5. Evaluation of the effects of *GH1* mutations on the structure and function of the GH molecule. This is particularly important for the assessment of those children with a clinical phenotype at the milder end of the clinical spectrum of short stature. In this group of patients, dysfunctional GH may be produced that is immunologically active and therefore falls within the normal range in GH function tests.

6. Development of rapid DNA diagnostic tests for inherited GH deficiency

7. Assessment of our postulate that GH deficiency is currently underdiagnosed and underestimated in the population.

Therefore, the characterisation of further, naturally occurring *GH1* lesions promises to be of considerable importance to studies of GH structure, function and expression. Studies of novel coding sequence variants should increase our understanding not only of GH function, but also of the interactions between GH and its receptor (GHR), and the process of GHR-mediated signal transduction. Insights obtained could be relevant to the rational design of a new generation of therapeutic agents. Similarly, studies of naturally-occurring *GH1* lesions in the promoter region should provide new insights into the control of *GH1* gene expression. Thus it may be seen that a broad spectrum of mutational lesions will necessarily improve our understanding of the relationship between mutant genotype and clinical phenotype in inherited forms of GH deficiency. Clearly, these studies are essential for the early detection and appropriate clinical management of familial GH deficiency.

The present invention therefore further provides a variant of *GH1*, which differs from *GH1* and is detectable by the method according to the invention but is not detectable by methods used hitherto, such as those reliant on patient selection criteria based primarily on height. Such *GH1* variants of the invention include those characterised in Example 6 and Table 7 hereinafter.

As indicated hereinbefore, current tests to assess GH secretion are many and varied and no single currently available investigation is ideal. Since the secretion of human GH is pulsatile, and because the amplitude and frequency of the GH pulses are extremely variable (being influenced by multiple internal and external factors

including sleep, exercise, stress and the pubertal stage of the individual concerned), those tests that yield the best information require close supervision of the patient in a dedicated investigation ward. The tests are therefore time-consuming, expensive, and cause considerable stress and distress to the patient and their family. The insulin-induced hypoglycaemic test (IST) is of particular note; it is used by many doctors, as mentioned above, to assess GH secretion but deaths have occurred owing to the treatment necessary for the hypoglycaemia induced in the patient as a necessary requirement of its successful implementation. It is therefore of paramount importance that the decision to perform an investigation, such as an IST, is most carefully considered before it is given a place in the assessment of a short child. The development of a DNA test for use in screening short patients would therefore have many advantages over the other tests currently available.

Accordingly, the present invention provides a screening method for screening a patient suspected of having dysfunctional GH, which screening method comprises the steps of:

(a) obtaining a test sample comprising a nucleotide sequence of the human *GH1* gene from the patient; and

(b) comparing a region of the sequence obtained from the test sample with the corresponding region of a predetermined sequence characterised in that the predetermined sequence is selected from a GH variant detectable according to the above-described method of the present invention.

More specifically, the screening method of the invention is characterised in that the predetermined sequence is an oligonucleotide having a nucleic acid sequence corresponding to a region of a variant GH1 gene, which region incorporates at least one variation when compared with the corresponding region of the wild type sequence.

Especially preferred is when the variation is one detectable by the detection method of the invention, such as any of those identified in Example 6 and Table 7 hereinafter.

1(a). PCR amplification of a 3.2 kb fragment containing the *GH1* gene in its entirety (promoter, five exons of the coding region, introns and untranslated regions) followed by the nested PCR of smaller, overlapping constituent fragments using primers designed so as to ensure *GH1* gene specificity. As well as using six known primers, the design of novel *GH1*-specific primers was found to be essential in order to avoid cross-contamination emanating from inadvertent PCR amplification of the paralogous, closely linked and highly homologous *GH2, CSH1* and *CSH2* gense, and the *CSHP1* pseudo-gene; and/or

1(b). PCR amplification of all or a fragment of genomic DNA spanning the Locus Control Region (hypersensitive sites I and II) approximately 15 kb upstream of the *GH1* gene of the patient [Jones *et al* Mol Cell Biol 15 7010-21 (1995)]. The Locus Control Region (LCR) is an enhancer region that affects the level and time of *GHT* transcription. The LCR is located ~14 kb 5' to the *GH1* gene and is responsible for the coordinate expression of the genes in the GH gene cluster. PCR amplification was carried out, using novel oligonucleotide primers, on two overlapping fragments (254 bp and 258 bp) in all patients;

2. Mutational screening of the *GH1* gene fragments by Denaturing High Performance Liquid Chromatography (DHPLC) using the Transgenomic WAVE ™System [O'Donovan *et al. Genomics* 52: 44-49 (1998)]. This screening method was selected for use since it is extremely rapid, cheap, sensitive and reproducible and exhibits, at least in our hands, a detection efficiency >95%. "Bandshifts" detected by DHPLC would represent potential DNA sequence variants; and

3. Characterisation of any such variants by DNA sequencing (either by automated or manual methods); and, optionally, but preferably also

4. Functional characterisation of *GH1* gene lesions using methodology appropriate to the location of the lesion and the inferred mechanism of dysfunction.

Therefore, the present invention further provides novel *GH1*-specific primers for use in the analysis of *GH1* as described above and in the examples, which primers include:

novel primers suitable for use in the DHPLC step (see Example 3, Table 6, for further details):

CTC CGC GTT CAG GTT GGC (GHD1F);
AGG TGA GCT GTC CAC AGG (GHD1R);
CTT CCA GGG ACC AGG AGC (GHD2R);
CAT GTA AGC CAA GTA TTT GGC C (GHD3F);
GGA GAA GGC ATC CAC TCA CGG (GHD4R);
TCA GAG TCT ATT CCG ACA CCC (GHD5F);
CGT AGT TCT TGA GTA GTG CGT CAT CG (GHD6R); and
TTC AAG CAG ACC TAC AGC AAG TTC G (GHD7F);

and primers suitable for use in the LCR step (all 5'→3', see also Example 5):

GTGCCCCAAGCCTTTCCC (LCR15: 1159-1177);
TGTCAGATGTTCAGTTCATGG (LCR13: 1391-1412);
CCTCAAGCTGACCTCAGG (LCR25: 1346-1363); and
GATCTTGGCCTAGGCCTCG (LCR23: 1584-1602).

The detection method of the invention and the GH variant identifiable or detectable thereby can give rise to the following additional advantages:

1. Expansion of the known spectrum of *GH1* gene mutations by identification and characterisation of new lesions.
2. Evaluation of the role of *GH1* gene mutations in the aetiology of short stature.
3. Identification of the mode of inheritance of novel *GH1* gene lesions.
4. Elucidation of the relationship between mutant genotype and clinical phenotype. This is deemed essential for the early detection and appropriate clinical management of GH deficiency.

respiratory conditions, such as severe asthma or cystic fibrosis; and skeletal problems, such as achondroplasia. A full medical history will also have been taken and used to complement the medical examination in order to aid the exclusion not only of the physical disorders identified above but also of psycho-social deprivation, another well-recognised cause of growth failure in childhood.

Optionally, the patient may also have been subjected to one or more growth hormone function tests. The term "growth hormone function tests" refers to tests of growth hormone secretion, such as those stimulation tests mentioned herinbefore, particularly the insulin-induced hypoglycaemic test (IST).

GH function tests are usually carried out on patients who are short; have been clinically assessed and had their height monitored over more than one visit to the endocrine clinic; have no other detectable cause for their growth failure; and therefore warrant being subjected to an assessment of their ability to produce growth hormone secretion from their pituitary gland following an appropriate stimulus, such as the profound drop in blood glucose that results from the administration of intravenous insulin.

Preferably, in the selection criteria for use in the detection method of this invention, a further criterion may also be applied in addition to (i) to (iii) above, namely:

(iv) growth failure, defined as a growth pattern [delineated by a series of height measurements; Brook CDG, Ed., *Clinical Paediatric Endocrinology*, 3rd Ed., (1995) Blackwell Science. Chapter 9, p141] which, when plotted on a standard height chart [Tanner *et al. Arch. Dis. Child* 45: 755-762 (1970)], predicts an adult height for the patient which is outside the patient's estimated target adult height range, the estimate being based upon the heights of the patient's parents.

Also useful as a reference is Tanner and Whitehouse. *Arch. Dis. Child* 51: 170-179 (1976)]. A patient's target adult height range is calculated as the mid-parental height (MPH) with the range being the 10th to 90th centile for MPH, which is sex-dependent:

MPH if male = [father's height + (mother's height +13)]/2 + or - in the range of from 6 to 8cm, usually 7.5cm; and

MPH if female = [(father's height - 13) + mother's height]/2 + or - in the range of from 6 to 8 cm, usually 6cm

These are standard tests and measurements used in the field and any other acceptable method of calculation can be used to determine growth failure, although the above-described method based on the description in Brook (*ibid,* 1996) regarding the formula to apply for predicting the limits of the target height range and on the description in Tanner (*ibid*, 1970) regarding the standard height charts are preferred according to this invention.

In the detection method according to this invention, therefore, although current height may be measured in order to apply the above-noted criteria, this is not in itself a criterion used for selection of patients in this method. As mentioned above, prior art methods rely on standard deviation from 'normal' height (*ie* absolute growth) as the criterion for selecting patients. The present invention does not require inclusion of such criterion and therefore the present invention provides a detection method in which absolute height is or may be excluded as a selection criterion.

Increasing the breadth of the *GH1* mutational spectrum will inevitably lead to a redefinition of inherited GH deficiency in molecular genetic terms. Furthermore, the recognition of novel types of short stature must eventually require the reclassification of GH deficiency as a disease entity. This will obviously have important implications for the screening and identification of individuals with short stature in whom the use of growth hormone treatment might be beneficial.

The test sample obtained from the patient in the detection method of the invention preferably comprises genomic DNA extracted from patient lymphocytes by standard procedures. *GH1* gene analysis is thereafter preferably carried out according to the following steps:

novel set of underlying mutational lesions. Some of these novel lesions could give rise to stable, yet dysfunctional, GH molecules that would exhibit normal immunological reactivity but little or no biological activity. On the basis of radio-immunoassay test results, dysfunctional GH molecules would have been erroneously regarded as normal. If such dysfunctional variants were to turn out to be common, then it would follow that GH deficiency is being under-diagnosed as a result of our current dependence on radio-immunoassay-based GH "function tests". Further, it would demonstrate an urgent need for the development of a true functional diagnostic assay.

We believe that height velocity is a more sensitive indicator of growth failure than absolute height measurements. The use of height velocity in conjunction with an assessment of bone age delay (retarded osseus maturation also due to GH deficiency); and other variables being normal, has allowed us to identify a unified group of patients with phenotypes which are less severe than that of classical IGHD patients having no GH, but who are more likely to have lesions of the *GH1* gene than those selected on the basis of height measurements alone.

Accordingly, the present invention provides a detection method for detecting a variation in *GH1* effective to act as an indicator of GH dysfunction in a patient, which detection method comprises the steps of:

(a) obtaining a test sample comprising a nucleotide sequence of the human *GH1* gene from the patient; and

(b) comparing the sequence obtained from the test sample with the standard sequence known to be that of the human *GH1* gene, wherein a difference between the test sample sequence and the standard sequence indicates the presence of a variation effective to act as an indicator of GH dysfunction (hereinafter "GH variant") characterised in that the test sample is obtained from a patient exhibiting the following cumulative criteria:

(i) height velocity below the 25th centile for age;

(ii) bone age delay according to the Tanner-Whitehouse scale of at least two years, when compared with chronological age; and

(iii) no other disorder known to cause inclusion in criteria (i) to (ii) above.

The present invention therefore further provides a GH variant detected by or detectable according to the above-described method of this invention.

With respect to the cumulative criteria (i) to (iii), each criterion may be assessed according to known methods and parameters readily available and described in the art, as elaborated further below:

(i) Tanner JM, Whitehouse RH. *Atlas of Children's Growth.* London: Academic Press, 1982; and Butler *et al. Ann. Hum. Biol.* 1990; 17: 177-198 (1990) are sources for statistics enabling a determination of the first criterion, *viz* that the height velocity of the patient is less than the 25th centile for the patient's age.

(ii) The Tanner-Whitehouse scale for assessing years of bone age delay is described by Tanner JM, Whitehouse RH, Cameron N *et al* in *Assessment of Skeletal Maturity and Prediction of Adult Height.* London: Academic Press, 1983.

(iii) Since short stature may also be secondary to conditions other than GH dysfunction, test samples from patients suffering from such disorders are excluded from the method of the invention. That the patient is suffering from no other disorder that might give rise to similar symptoms to that of GH dysfunction is determined by baseline investigations. "Baseline investigations" therefore include tests to exclude, particularly, hypothyroidism; pseudo-hypoparathyroidism; malabsorption syndromes *eg* coeliac disease; renal and hepatic diseases; haematological disorders, such as anaemia; and a karyotype to check that a chromosome disorder such as Turner syndrome is not the cause of the growth failure. The patient may also have had a thorough clinical examination in order to exclude other causes of growth failure, for example, cardiac disease including congenital heart disease; chronic auto-immune conditions, such as rheumatoid arthritis and inflammatory bowel disease; chronic

	(ii) *CSH1, GH2, CSH2* (~32kb)	Homozygous. Consanguinous marriage.	

The gene encoding growth hormone (*GH1*) was one of the first human genes to be cloned and the first gross gene deletions (6.7kb type) responsible for inherited growth hormone deficiency were soon detected by Southern blotting. All gross deletions involving the *GH1* gene result in severe (type IA) deficiency, characterised by the total absence of GH. ~70% of characterised deletions of the *GH1* gene are 6.7 kb in length, whilst most of the remainder are of 7.6 kb or 7.0 kb (Table 2B).

In addition, several examples of much more infrequent deletions have been reported.In recent years, various attempts have been made to move away from Southern blotting toward PCR-based approaches as a mutation screening tool. Homozygous *GH1* gene deletions have been fairly readily detected by PCR amplification of the *GH1* gene and flanking regions followed by restriction enzyme digestion of the resulting PCR products. Although this approach has been used successfully to exclude homozygosity for a *GH1* gene deletion in at-risk pregnancies, it is however unable to distinguish homozygosity for the wild-type gene from heterozygosity for a gene deletion. It would also fail to detect deletions other than the relatively short 6.7, 7.0 and 7.6kb deletions that remove only the *GH1* gene.

PCR primers have been designed which immediately flank the *GH1* gene and which generate a 790bp fragment from control DNA samples. Absence of this fragment was held to be indicative of a *GH1* gene deletion but the use of "non-specific PCR fragments" as internal controls for PCR amplification must make the reliability of this method somewhat suspect.

Table 3 Micro-deletions in the *GH1* gene causing GH deficiency and short stature

Deficiency type	**Deletion (Lower case letters denote the deleted bases.** ^	**Codon (Numbering is**	**Post-treatment**

	specifies the location of the numbered codon immediately downstream.)	relative to translational initiation codon ATG at -26.)	antibodies present?
IA	GCCTG^CTCTGcCTGCCCTGGC	-11	yes
II	CCCCAGGCGGgatgggggagacctgtaGTC AGAGCCC	Intron 3 (del+28 to +45)	no
IA	TCTGT^TTCTCagAGTCTATTCC	54	no

As well as gross deletions, three micro-deletions of the *GH1* gene have been reported; two of these patients were also heterozygous for the 6.7 kb *GH1* gene deletion (Table 3).

Only six different single base-pair substitutions have been reported from within the coding region of the *GH1* gene (Table 4). Two of these are nonsense mutations converting amino acid residues Trp-7 and Glu-4 in the signal peptide to stop codons. These mutations are the only known *GH1* gene lesions to cause type IA deficiency that are not gene deletions. Since these lesions predict termination of translation within the signal peptide, they would be incompatible with the production of a functional GH molecule. The other four single base-pair substitutions are missense mutations that result in the production of dysfunctional growth hormone molecules.

Table 4 Single base-pair substitutions in the *GH1* coding region causing GH deficiency and short stature

Deficiency type	Nucleotide substitution	Amino acid substitution	Codon (numbering relative to translational initiation codon ATG at -26)	Post-treatment antibodies present?
IA	ACA→GCA	Thr→Ala	-24	no
IA	TGG→TAG	Trp→Term	-7	no

IA	GAG→TAG	Glu→Term	-4	yes
II	CGC→TGC	Arg→Cys	77	no
?	GAC→GGC	Asp→Gly	112	no
?	CGC→CAC	Arg→His	183	no

Single base-pair substitutions in the promoter region of possible pathological significance were first sought by sequencing the promoter region of the *GH1* gene (between -60 and +70 relative to the transcriptional initiation site) in three Chinese patients with IGHD IA and 2 controls. Several differences were noted but these were probable polymorphisms and were not characterised further. As mentioned above, the promoter region of the *GH1* gene has subsequently been shown to exhibit a very high level of sequence polymorphism with 17 variant nucleotides within a 570 bp stretch (Figure 3). However, they were not found to be over-represented in patients as compared to controls.

GH1 promoter variation has also been separately investigated and a total of 22 variant polymorphic sites were detected, mostly single base-pair substitutions: 17 of these occurred in a 550 bp region 5' to the ATG initiation codon, three occurred around position -1075 5' to ATG, and two occurred within intron 1 (IVS1) at positions 76 and 219 respectively [Wagner *et al*, Eur J Endocrinol 137 474-81 (1997)]. All except four of these variants were also noted in controls but these four variants were not considered to be the cause of the growth hormone deficiency. Only one of the variant sites occurred within a sequence homologous to a transcription factor binding site: the alternative presence of CCAGA and GAGAG sequences at -333 within a potential (but not proven) NF-1 binding site.

Therefore, to date, no mutations of pathological significance have been reported in the *GH1* gene promoter.

Single base-pair substitutions affecting mRNA splicing have also been described in the *GH1* gene. Most are associated with a comparatively rare dominant form of GH deficiency (Table 5).

The transversions in the intron 4 donor splice site have been shown by mRNA *in vitro* expression analysis of transfected cells to activate a cryptic splice site within exon 4, 73bp 5' to the exon 4 donor splice site. This would predict the generation of an aberrantly spliced product lacking amino acids 103-126 encoded by exon 4 and, as a consequence of a shift in the reading frame, the incorporation of 94 novel amino acids including 29 resulting from read-through of the normally untranslated 3' non-coding region of the *GH1* gene.

Table 5 Single base-pair substitutions affecting mRNA splicing and causing GH deficiency and short stature

Deficiency type	Nucleotide substitution/ position	Splice site	Ethno-geographic origin/zygosity
II	G→A, +1	IVS3 donor	Sweden, North America, Northern Europe, South Africa, Chile/heterozygous
II	G→C, +1	IVS3 donor	Turkish/ heterozygous
II	G→A, +5	IVS3 donor	Chilean/ heterozygous
II	G→C, +5	IVS3 donor	?
II	T→C, +6	IVS3 donor	Turkish/ heterozygous Asian/ heterozygous
II	G→A, +28	IVS3 donor	?/heterozygous
IB	G→C, +1	IVS4 donor	Saudi Arabian/ homozygous
IB	G→T, +1	IVS4 donor	Saudi Arabian/ homozygous
IB	G→C, +5	IVS4 donor	?

Since the region of the GH protein encoded by exons 4 and 5 is thought to be important for correct targeting of the protein to secretory granules, it has been predicted that this aberrant protein would not be secreted normally. However, no

antibodies to exogenous GH have been noted in patients with type IB GH deficiency. The avoidance of immune intolerance may thus indicate that at least some of the aberrant protein product could be secreted and that it could be partially stable in the circulation. By contrast, the five known splicing mutations within IVS3 (Table 5) are associated with a type II deficiency state manifesting autosomal dominant inheritance through the affected families.

GH deficiency patients with truncating *GH1* mutations or homozygous gene deletions are at considerable risk of developing anti-GH antibodies upon GH treatment. By contrast, we are not aware of any reports describing allo-antibody formation in patients with either missense mutations or single base-pair substitutions within splice sites.

Until now, no other correlations between mutant genotype and clinical phenotype have been reported. The requisite data in the published literature are sparse and very variable in quality, but we have attempted a crude meta-analysis as a means of gauging whether or not patients with gross gene deletions differ from patients with splice site mutations in terms of their clinical and phenotypic sequelae. The height of the patients with *GH1* deletions was found to be on average 7.3 SD below the age-adjusted mean (n=29), as compared with an average of 5.4 SD below the mean (n=17) for the patients with *GH1* splicing mutations. Although bone age delay was greater and growth velocity lower in the deletion patients, such findings are very difficult to interpret since they may be subject to bias of ascertainment.

Since most cases of familial GH deficiency hitherto described are inherited as an autosomal recessive trait, some examples of the inherited deficiency state are likely to have gone unrecognized owing to small family size. Similarly, cases of GH deficiency resulting from *de novo* mutations of the *GH1* gene could be classified as sporadic, and a genetic explanation for the disorder would neither be entertained nor sought. Finally, depending upon the criteria used for defining the deficiency state, it may be that the full breadth of both the phenotypic and genotypic spectrum of GH deficiency may never have come to clinical attention. For these reasons, current estimates of the

prevalence of GH deficiency could be inaccurate and may therefore seriously underestimate the true prevalence in the population.

The definition of IGHD favoured by many combines (a) severe growth retardation, often - as mentioned above - defined as <-4.5 SD in height; (b) reduced GH response to stimulation/provocation (*ie* a serum GH level of <4ng/ml); and (iii) no other cause for growth retardation. The strict adherence to formal definitions of what constitutes GH deficiency and the fairly uniform acceptance of these criteria, especially criterion (b) in selecting patients for study [Shalet SM *et al. Endocrine Rev.* 19: 203-223 (1998)] would have served to ensure that the described *GH1* mutational spectrum was not only far from complete but also unrepresentative of the wider mutational spectrum. Thus, mutations responsible for GH deficiency states in which the SD scores were less severe or the GH levels less reduced (*eg* missense mutations within the coding region of the gene or promoter mutations) would have been much less likely to come to clinical attention. Indeed, this may go some way toward explaining why only four different missense mutations have so far been reported in the *GH1* gene, a finding which is virtually unprecedented for a fairly prevalent disorder that has been studied at the molecular level for nearly 20 years [Krawczak *et al*, Hum. Mutation 15, 45-51 (2000)].

The complete absence of GH produces a readily recognisable and severe clinical phenotype that has been extensively studied. In those reported studies in which the phenotype of the patients is less severe and in which patient selection criteria have actually been identified, patient ascertainment strategies have generally used the deviation of an individual's height from the mean height for their age as a diagnostic indicator of growth failure.

The selection of patients using criteria (a) and (b), as defined above, will serve to define patients with a severe degree of IGHD-related growth failure. We have proposed that moderating the criteria applied in selecting patients for study would be likely to lead to the inclusion of patients whose growth failure is a manifestation of a different portion of the GH deficiency spectrum, and which could therefore yield a

TTC AAG CAG ACC TAC AGC AAG TTC G (GHD7F).

8. A method according to claim 7, wherein the test sample is obtained from a patient exhibiting the following cumulative criteria:

(i) height velocity below the 25th centile for age;

(ii) bone age delay according to the Tanner-Whitehouse scale of at least two years when compared with chronological age; and

(iii) no other disorder known to cause inclusion in criteria (i) to (ii) above.

9. A method according to claim 7 or 8, wherein the detection method comprises PCR amplification of the *GH1* gene of the patient.

10. A method according to claim 9, wherein the detection method comprises PCR amplification of the entire *GH1* gene of the patient and nested PCR of overlapping constituent fragments of the *GH1* gene of the patient.

11. A method according to claim 7 ,8 or 9, wherein the detection method comprises PCR amplification of all or a fragment of genomic DNA spanning the Locus Control Region.

12. A GH variant detected by or detectable by a method according to any preceding claim.

13. A variant of *GH1*, which differs from *GH1* and is detectable by a method according to any preceding claim .

14. A variant of *GH1*, which differs from *GH1* and is detectable by a method according to any preceding claim, which variant is not detectable by methods wherein the patient selection criteria are based primarily on height and do not include the cumulative criteria (i) to (iii) as defined in claims 1, 2 and 8.

15. A GH variant or a variant of *GH1* according to any of claims 12to 14, which variant is selected from those characterised as unpublished in Example 6 and Table 7 herein.

16. A screening method for screening a patient suspected of having dysfunctional GH or dysfunctional GH axis, which screening method comprises the steps of:
(a) obtaining a test sample comprising a nucleotide sequence of the human *GH1* gene from the patient; and
(b) comparing a region of the sequence obtained from the test sample with the corresponding region of a predetermined sequence
wherein the predetermined sequence is selected from a *GH1* variant detectable by a method according to any of claims 1 to 11.

17. A screening method according to claim 16, wherein the predetermined sequence is an oligonucleotide having a nucleic acid sequence corresponding to a region of a variant *GH1* gene, which region incorporates at least one variation when compared with the corresponding region of the wild type sequence.

18. A screening method according to claim 16 or claim 17, wherein the variation is or includes any of those characterised as unpublished in Example 6 and Table 7 herein.

19. A screening method according to any of claims 16 to 18, wherein the test sample comprises genomic DNA.

20. A screening method for determining GH dysfunction, comprising:
(a) obtaining a first human tissue suspected of GH dysfunction; and
(b) comparing the *GH1* gene or *GH1* transcript, or fragment therefrom (*eg* cDNA), in the first human tissue to the corresponding gene, transcript or fragment of a *GH1* variant obtainable from a second human tissue derived from a human exhibiting the following cumulative criteria:
(i) height velocity below the 25th centile for age;

CLAIMS

1. A detection method for detecting a variation in *GH1* effective to act as an indicator of GH dysfunction in a patient, which detection method comprises the steps of:

(a) obtaining a test sample comprising a nucleotide sequence of the human *GH1* gene from the patient; and

(b) comparing the sequence obtained from the test sample with a standard sequence known to be that of the human *GH1* gene, wherein a difference between the test sample sequence and the standard sequence indicates the presence of a variation effective to act as an indicator of GH dysfunction (hereinafter "GH variant");

wherein the test sample is obtained from a patient exhibiting the following cumulative criteria:

(i) height velocity below the 25th centile for age;

(ii) bone age delay according to the Tanner-Whitehouse scale of at least two years when compared with chronological age; and

(iii) no other disorder known to cause inclusion in criteria (i) to (ii) above.

2. A method according to claim 1,

wherein the test sample is obtained from a patient exhibiting the following cumulative criteria:

(i) height velocity below the 25th centile for age;

(ii) bone age delay according to the Tanner-Whitehouse scale of at least two years when compared with chronological age;

(iii) no other disorder known to cause inclusion in criteria (i) to (ii) above; and

(iv) growth failure, defined as a growth pattern [delineated by a series of height measurements; Brook CDG, Ed., *Clinical Paediatric Endocrinology*, 3rd Ed., (1995) Blackwell Science. Chapter 9, p141] which, when plotted on a standard height chart [Tanner *et al. Arch. Dis. Child* 45: 755-762 (1970)], predicts an adult height for the patient which is outside the patient's estimated target adult height range, the estimate being based upon the heights of the patient's parents.

3. A method according to claim 1 or claim 2, wherein the detection method comprises PCR amplification of the *GH1* gene of the patient.

4. A method according to claim 3, wherein the detection method comprises PCR amplification of the entire *GH1* gene of the patient and nested PCR of overlapping constituent fragments of the *GH1* gene of the patient.

5. A method according to claim 3 or claim 4, wherein the detection method comprises PCR amplification of all or a fragment of genomic DNA spanning the Locus Control Region.

6. A method according to any preceding claim, wherein the detection method comprises mutational screening of the patient's *GH1* gene fragments by DHPLC.

7. A detection method for detecting a variation in *GH1* effective to act as an indicator of GH dysfunction in a patient, which detection method comprises the steps of:

(a) obtaining a test sample comprising a nucleotide sequence of the human *GH1* gene from the patient; and

(b) comparing the sequence obtained from the test sample with a standard sequence known to be that of the human *GH1* gene, wherein a difference between the test sample sequence and the standard sequence indicates the presence of a variation effective to act as an indicator of GH dysfunction (hereinafter "GH variant");

which detection method further comprises the use of one or more primer(s) selected from:

CTC CGC GTT CAG GTT GGC (GHD1F);
AGG TGA GCT GTC CAC AGG (GHD1R);
CTT CCA GGG ACC AGG AGC (GHD2R);
CAT GTA AGC CAA GTA TTT GGC C (GHD3F);
GGA GAA GGC ATC CAC TCA CGG (GHD4R);
TCA GAG TCT ATT CCG ACA CCC (GHD5F);
CGT AGT TCT TGA GTA GTG CGT CAT CG (GHD6R); and

(ii) bone age delay according to the Tanner-Whitehouse scale of at least two years when compared with chronological age; and

(iii) no other disorder known to cause inclusion in criteria (i) to (ii) above; and, optionally,

(iv) growth failure defined as a growth pattern [delineated by a series of height measurements; Brook CDG, Ed., *Clinical Paediatric Endocrinology*, 3rd Ed., (1995) Blackwell Science. Chapter 9, p141] which, when plotted on a standard height chart [Tanner *et al. Arch. Dis. Child* 45: 755-762 (1970)], predicts an adult height for the patient which is outside the patient's estimated target adult height range, the estimate being based upon the heights of the patient's parents.

21. A kit suitable for use in carrying out a screening method according to any of claims 16 to 20, which kit comprises:

(a) an oligonucleotide having a nucleic acid sequence corresponding to a region of a variant *GH1* gene, which region incorporates at least one variation from the corresponding wild-type sequence; and

(b) an oligonucleotide having a nucleic acid sequence corresponding to the wild-type sequence in the region specified in (a); and, optionally,

(c) one or more reagents suitable for carrying out PCR for amplifying desired regions of the patient's DNA.

22. A kit according to claim 21, wherein kit component (a) comprises a plurality of said oligonucleotides immobilised on a solid support.

23. A composition comprising a GH variant or a variant of *GH1* according to any of claims 12 to 15, in association with a pharmaceutically acceptable carrier therefor.

24. An isolated, purified or recombinant nucleic acid sequence selected from:

(a) a sequence encoding a variant of *GH1* according to any of claims 13 to 15;

(b) a sequence substantially homologous to or that hybridises to sequence (a) under stringent conditions; or

(c) a sequence substantially homologous to or that hybridises under stringent conditions to the sequence (a) or (b) but for the degeneracy of the genetic code; or
(d) an oligonucleotide specific for any of the sequences (a), (b) or (c).

25. A vector comprising a nucleic acid sequence according to claim 24.

26. A host cell comprising a vector according to claim 25, such as a bacterial host cell.

27. A process for preparing a variant of *GH1* according to any of claims 13 to 15, which process comprises:
(i) culturing a host cell according to claim 26; and
(ii) recovering from the culture medium the variant of *GH1* thereby produced.

28. A protein or amino acid sequence encoded or expressed by a sequence, vector, cell or culture medium as defined in any of claims 24 to 27.

ABSTRACT

SEQUENCES

A detection method for detecting a variation in *GH1* effective to act as an indicator of GH dysfunction in a patient, comprises the steps of comparing a test sample comprising a nucleotide sequence of the human *GH1* gene from the patient with a standard sequence known to be that of the human *GH1* gene. A difference between the test sample sequence and the standard sequence indicates the presence of a variation effective to act as an indicator of GH dysfunction (hereinafter "GH variant"). The test sample is obtained from a patient exhibiting the following cumulative criteria:

(i) height velocity below the 25th centile for age;

(ii) bone age delay according to the Tanner-Whitehouse scale of at least two years when compared with chronological age; and

(iii) no other disorder known to cause inclusion in criteria (i) to (ii) above.

Also disclosed are mutations thereby detected, and their use in screening patients for growth hormone irregularities or for producing variant proteins suitable for treating such irregularities.

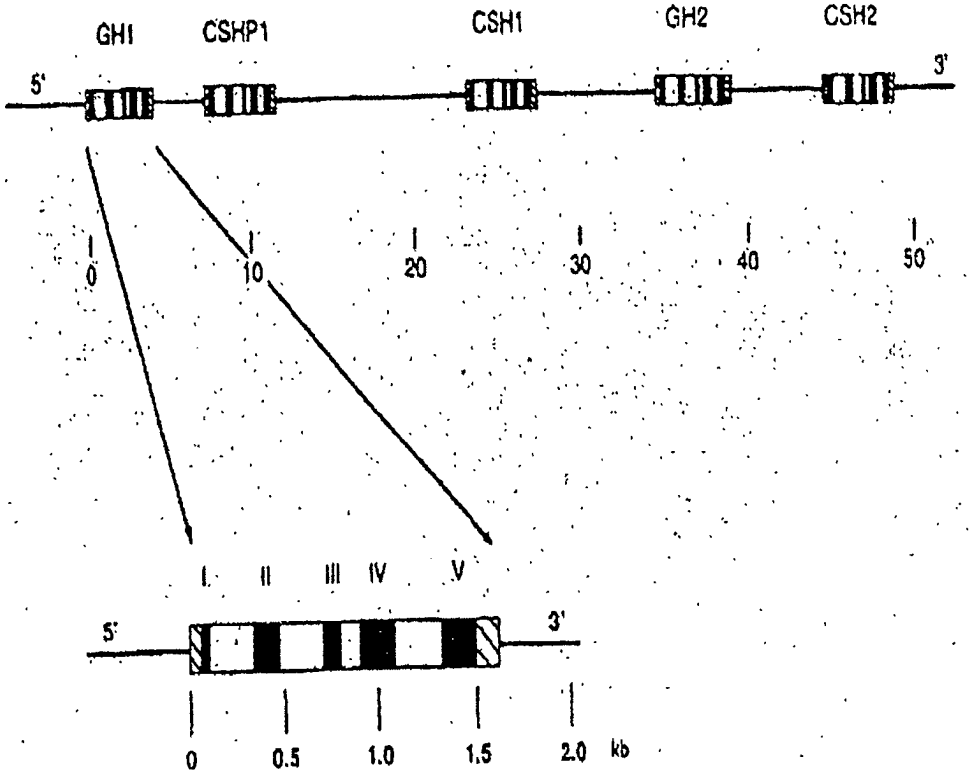

Fig. 1 Human growth hormone gene cluster on chromosome 17q23. The fine structure of the *GH1* gene is shown below. The scales are in kilobases (kb)

21+

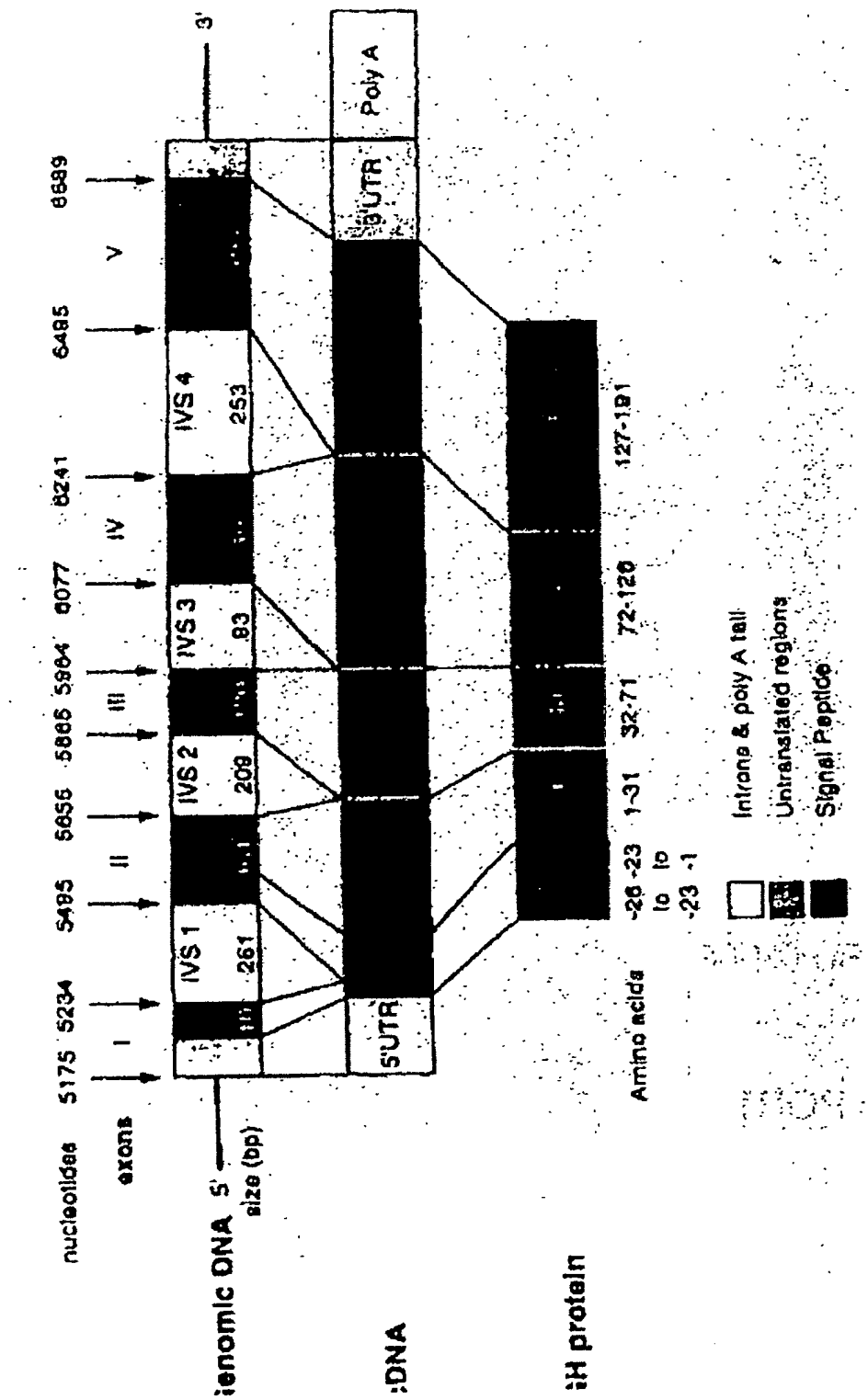
nucleotides
exons
size (bp)
genomic DNA 5'
3'
5175
5234
5485
5656
5886
5984
6077
6241
6485
6689
I
II
III
IV
V
IVS 1
261
IVS 2
209
IVS 3
83
IVS 4
253
5'UTR
Poly A
:DNA
H protein
Amino acids
-26 -23
to
-23 -1
1-31
32-71
72-120
127-191
Introns & poly A tail
Untranslated regions
Signal Peptide

Figure 2

													IVS4 T → A 1169
59 (5)	T	A	G	G	G	A	G	A	A	T	ND		IVS4 T → A 1169
60 (2)	T	A	G	-	G	A	G	A	A	T	Acceptor splice site IVS2 G → A at –1 (702)	Unpublished	IVS4 T → A 1169
60 (4)	T	A	G	G	G	A	G	A	A	T	ND		IVS1 T → C 281 IVS4 T → A 1169
61 (1)	T	A	T	G	A	A	G	A	A	T	ND		ND
61 (4)	T	A	G	-	G	A	G	A	A	T	Acceptor splice site IVS2 G → A at –1 (702)		ND
62 (1)	T	A	G	G	G	A	G	A	A	T	ND		IVS4 T → A 1169
63													
64 (7)	T	G	G	G	G	A	G	A	A	T			
64 (8)	T	A	T	G	A	A	G	A	A	T			
65 (1)	T	A	T	G	A	A	G	A	A	T	A → G -248	Unpublished	IVS4 T → A 1169
67 (13)	T	A	G	G	G	A	G	C	A	T			
67 (15)	T	A	G	G	G	A	G	A	A	T			
68 (7)	T	A	T	G	A	A	G	A	A	T			
69 (3)	T	A	T	G	A	A	G	A	A	T			
69 (11)	T	A	T	G	A	A	G	A	A	T	Glu30Gly (GAG → GGG. 489)		Tyr103 (TAC → TAT: 1010) IVS4 T → G 1196
70 (5)	T	A	T	G	A	A	G	A	A	T	T → C -30		ND
70 (10)	T	G	G	G	G	A	G	G	C	T	A → G -267 A → G -248 Gln22Arg (CAG → CGG: 465) Lys41Arg (AAG → AGG: 731) Trp86Arg (TGG → CGG: 957)		IVS4 T → A 1169 3'UTR T → C 1654

Key:
IVS: intervening sequence (intron)
ND: no mutation or polymorphism detected
UTR: untranslated region
[a] Patient (clone number)
[b] Nucleotide numbering based on GH1 reference sequence. At -31, alternative alleles are presence or absence of a G.
[c] Amino acid residue number and substitution, (nucleotide substitution and number based upon *GH1* reference sequence).
[d] IVS number, nucleotide change, base number
[e] Miyata I, Cogan J, Prince MA, Kamijo T, Ogawa M, Phillips JA (1997) Detection of growth hormone defects by dideoxy fingerprinting (ddF). *Endocrinol J.* 44: 149-154.

receptor to enhance the proliferation and maturation of many tissues, including bone, cartilage, and skeletal muscle. In addition to promoting growth of tissues, GH has also been shown to exert a variety of other biological effects, including lactogenic, diabetogenic, lipolytic and protein anabolic effects, as well as sodium and water retention.

Adequate amounts of GH are needed throughout childhood to maintain normal growth. Newborns with GH deficiency are usually of normal length and weight. Some may have a micropenis or fasting hypoglycemia in conjunction with low linear postnatal growth, which becomes progressively retarded with age. In those with isolated growth hormone deficiency (IGHD), skeletal maturation is usually delayed in association with their height retardation. Truncal obesity, facial appearance younger than expected for their chronological age and delayed secondary dentition are often present. Skin changes similar to those seen in premature ageing may be seen in affected adults.

Familial IGHD comprises several different disorders with characteristic modes of inheritance. Those forms of IGHD known to be associated with defects at the *GH1* gene locus are shown in Table 1 together with the different types of underlying lesion so far detected.

Table 1 Classification of inherited disorders involving the *GH1* gene

Disorder	Mode of inheritance	Types of gene lesion responsible	GH protein	Deficiency state
IGHD IA	Autosomal recessive	Gross deletions, micro-deletions, nonsense mutations	Absent	Severe short stature. Anti-GH antibodies often produced upon GH treatment, resulting in poor response thereto.
IGHD IB	Autosomal recessive	Splice site mutations	Deficient	Short stature. Patients usually respond well to exogenous GH
IGHD II	Autosomal dominant	Splice site and intronic mutations, missense mutations	Deficient	Short stature. Patients usually respond well to exogenous GH